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Hepatitis C Virus is Frequently Coinfected With Serum Marker–Negative Hepatitis B Virus: Probable Replication Promotion of the Former by the Latter as Demonstrated by In Vitro Cotransfection

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Patients with hepatitis C have been reported occasionally to be coinfected with serum marker-negative (silent) hepatitis B virus (HBV). The frequency and significance of such coinfection were investigated. Thirty patients with hepatitis C virus (HCV) infections (10 acute, 10 chronic, 10 cirrhotic) were selected randomly; the acute cases were without serum hepatitis B surface antigen (HBsAg) and anti-hepatitis B core IgM, and the chronic cases were without HBsAg. A nested polymerase chain reaction for the X open reading frame was used to amplify HBV DNA in serum, and immunoperoxidase staining was carried out on liver biopsy specimens. Nucleotide sequencing was carried out to characterize the amplified HBV DNAs. In order to clarify the possibility that the silent HBV mutant promotes HCV replication in the liver, the full-length HCV RNA and the cloned silent HBV DNA dimer were cotransfected into an established cell line, HuH-7, and the amount of secreted HCV RNA was quantified serially. The target HBV DNA was amplified in 26 (86.7%) of the 30 patients. Subsequent direct nucleotide sequencing in 9 selected patients revealed an 8-nucleotide deletion, characteristic of a silent HBV mutant. Immunostaining revealed hepatitis B surface antigen in 15 (50.0%). Cotransfected silent HBV DNA augmented the secretion of HCV RNA by up to 5-fold in comparison with HCV RNA transfection alone. In conclusion, HCV is coinfected frequently with the silent HBV mutant and the latter probably promotes the replication of the former in the liver. ***J. Med. Virol. 52:399–405, 1997.***

KEY WORDS: hepatitis C virus; hepatitis B virus; mutation; X gene; transfection

INTRODUCTION

The complete nucleotide (nt) sequence of serum marker-negative (silent) hepatitis B virus (HBV) recovered from patients with acute hepatitis negative for hepatitis B surface antigen (HBsAg) and anti-hepatitis B core (anti-HBc) IgM in acute hepatitis, and patients with chronic hepatitis negative for HBsAg, has been described [Uchida et al., 1994a; Uchida et al., 1995]. These HBV DNAs are characterized by an 8-nt deletion in the region encoding the X-gene. This mutation is thought to suppress the replication and expression of HBV DNA, leading to non-detectability of the above serum immunoserological markers. The results were confirmed recently by another laboratory [Fukuda et al., 1996]. Silent HBV infection has also been reported outside Japan [Brechot et al., 1991; Feitelson et al., 1994; Liang et al., 1991; Preisler-Adams et al., 1993], and some of the HBV DNAs in these cases were shown to have deletions of various lengths within the X gene [Feitelson et al., 1994; Preisler-Adams et al., 1993].

Coinfection of silent HBV with hepatitis C virus (HCV) has been reported previously [Baginski et al., 1992; Brechot et al., 1991; Chung et al., 1995; Gonzalez et al., 1995; Sardo et al., 1994]. According to these reports, HBV DNA was detected by polymerase chain reaction (PCR) in 12 (28.6%) of 42 liver samples [Sardo et al., 1994] and 37 (53.6%) of 69 serum samples [Gonzalez et al., 1995] from patients with chronic hepatitis C. In Japan there has been no systematic analysis of such coinfection. We report a high frequency of coinfection in Japanese patients demonstrated by PCR and by immunostaining. Furthermore, we address the hy-

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TABLE I. Clinical Summary of Patients and Results of Immunostaining

Patient #	Age (y)	Sex	HBsAg/ Ab	HBcAb/ HBcAb IgM	HBeAg/ Ab	HCV RNA	HCV genotype	Bilirubin (mg/dl)	ALT/AST	Transf	Immunostaining HCV	HBsAg	HBcAg
Acute hepatitis													
1	60	M	−/+	−/−	−/−	+	2a	0.7	215/146	−	+	−	−
2	36	M	−/−	−/−	−/−	+	1b	1.2	178/214	+	+	−	−
3	20	F	−/−	−/−	−/−	+	1b	0.8	789/541	+	+	−	−
4	54	F	−/−	NT/−	NT/NT	+	1b	1.0	243/301	+	+	−	−
5	50	M	−/−	NT/−	−/−	+	1b	1.0	83/60	+	+	−	−
6	31	F	−/−	−/−	−/−	+	1a + 1b	17.2	503/692	+	−	−	−
7	73	M	−/−	NT/−	−/−	+	1b	0.8	86/111	+	+	−	−
8	47	F	−/−	−/−	NT/NT	+	2b	3.4	1745/171	+	−	−	−
9	38	F	−/−	NT/−	NT/NT	+	2a	2.1	483/420	+	+	−	−
10	24	M	−/−	−/−	−/−	+	2b	5.4	491/372	+	+	−	−
Chronic hepatitis													
11	27	M	−/−	−/NT	NT/NT	+	2a	1.0	102/65	−	−	−	−
12	58	F	−/−	−/NT	NT/NT	+	1b	0.8	31/39	+	+	+	−
13	58	F	−/−	+/NT	NT/NT	+	1b	0.8	24/27	+	−	−	−
14	45	M	−/+	+/NT	−/+	+	1b	0.9	107/72	−	+	+	−
15	60	F	−/+	+/NT	−/+	+	1b	0.7	35/49	+	−	−	−
16	56	M	−/−	+/NT	−/−	+	1b	0.4	19/40	−	−	+	+
17	67	M	−/−	−/NT	−/−	+	2a	1.0	62/51	−	+	+	−
18	67	M	−/−	−/NT	−/−	−	NT	0.9	11/23	−	−	−	−
19	28	M	−/−	−/NT	−/−	+	1b	0.7	136/10	−	−	−	−
20	60	M	−/−	−/NT	NT/NT	+	1b	1.0	96/67	−	+	+	−
Liver cirrhosis													
21	62	F	−/+	+/NT	−/+	−	NT	2.2	11/34	−	+	+	+
22	42	M	−/+	+/NT	−/+	+	1b	1.6	57/89	+	+	+	−
23	57	M	−/+	−/NT	NT/NT	+	2a	0.8	106/12	+	+	+	+
24	50	F	−/−	−/NT	−/−	+	1b	0.9	88/94	+	+	+	−
25	60	M	−/−	−/NT	−/−	+	1b	0.5	56/62	−	+	+	−
26	60	F	−/−	−/NT	NT/NT	+	2a	0.7	78/66	+	+	+	+
27	61	M	−/−	−/NT	−/−	+	1b	1.8	14/52	+	+	+	−
28	58	M	−/−	−/NT	−/−	+	1b	0.7	147/13	−	+	+	−
29	70	M	−/−	−/NT	−/−	+	1b	5.4	35/82	+	+	+	−
30	46	M	−/+	+/NT	−/+	+	1b	1.4	74/92	−	+	+	−

Bilirubin and ALT are at peak for acute hepatitis and at the time of biopsy for chronic hepatitis and cirrhosis. HBsAg/Ab, HBcAg/Ab, and HBeAg/Ab are at the time of admission.
HBsAg/Ab, hepatitis B surface antigen/anti-hepatitis B surface; HBcAg/Ab, hepatitis B core antigen/anti-hepatitis B core; HBeAg/Ab, hepatitis B e antigen/anti-hepatitis B e; ALT/AST, serum alanine aminotransferase/aspartate aminotransferase (IU/L); Transf, history of transfusion; HCV, hepatitis C virus; HCV RNA, hepatitis C virus RNA as determined by polymerase chain reaction after reverse transcription in the 5′-noncoding region; HCV genotype, tested according to the procedure described [Okamoto et al., 1992]; NT, not tested.

pothesis that silent HBV promotes HCV replication in the light of data obtained by in vitro cotransfection.

MATERIALS AND METHODS

Patients

Thirty Japanese patients (aged between 24 and 73; 19 men and 13 women) with hepatitis C who were admitted to Hokkaido Kin-ikyo Hospital were selected at random. The clinicopathological diagnosis was acute hepatitis, chronic hepatitis, and liver cirrhosis in 10 patients in each category. The patients with acute hepatitis were admitted between January 1985 and January 1994, and the other patients between January 1994 and April 1994. Nine of the 10 patients with acute hepatitis subsequently developed chronic hepatitis; one of the 30 patients died. Eighteen had a past history of blood transfusion, but the probable route of infection in the other 12 was unclear. A summary of the clinical and laboratory data is given in Table I.

All 30 patients had anti-HCV, and 28 had serum HCV RNA, as demonstrated by PCR after reverse transcription in the 5′-noncoding region. All 30 patients were negative for HBsAg. The patients with acute hepatitis were negative for anti-hepatitis A antibody and anti-HBc IgM. Seven (23.3%) of the 30 patients had anti-HBs. Seven (26.9%) of 26 patients tested were positive for anti-HBc and 5 (23.8%) of 21 patients had anti-hepatitis Be. All these tests for serological viral markers were carried out at the time of admission. The immunoserological viral marker data for individual patients are shown in Table I. The viral markers were tested by radioimmunoassay (Dainabot Co. Ltd., Japan), except for anti-HCV, which was tested by an enzyme-linked immunosorbent assay (second generation, Dainabot Co., Ltd.). The HCV genotype was examined according to the procedure described previously [Okamoto et al., 1992] and categorized according to the classification of Simmonds et al. [1993].

Polymerase Chain Reaction and Nucleotide Sequencing

The patients' sera were subjected to PCR at the time of admission for acute hepatitis, and at the time of biopsy for chronic infection. A 100-μl aliquot of each

serum sample was mixed with 300 μl of lysis buffer consisting of 12.0 mM Tris-hydrochloride buffer, pH 8.0, 10.0 mM EDTA, 0.6% sodium dodecyl sulfate, and 120 μg/ml proteinase K, and incubated at 70°C for 3 hr. DNA was extracted with phenol/chloroform and dissolved in 100 μl of water after ethanol precipitation.

Nested PCR for the X open reading frame (ORF) was carried out using 5 μl of the DNA extract as a template, each of the primers at 0.25 μM, and 1 unit of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) in PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 0.002% gelatin) as well as each dNTP at 100 μM in a total volume of 100 μl. The mixtures were processed through 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min in a programmable heat block (Perkin-Elmer Cetus). The primer pair was P197 5′CCATACTGCGGAACTCCTAG3′ (at nt 1268 to 1287) and P201 5′ATTAGGCAGAGGTGAAAAAG3′ (at nt 1822 to 1841) for the first-stage PCR and P198 5′TTTTGCTCGCAGCCGGTCTG3′ (at nt 1295 to 1314) and P201 for the second-stage PCR. Each PCR included 5 negative controls.

After confirmation that the bands were of the expected size using a minigel, the products of the second PCR for 9 patients (Nos. 8–10 with acute hepatitis, Nos. 17, 19, and 20 with chronic hepatitis, and Nos. 28–30 with liver cirrhosis) were electrophoresed through a 2% agarose gel and the DNAs were purified using a Geneclean II kit (BIO 101 Inc.; La Jolla, CA).

The purified DNAs were then dissolved in 20 μl of water and a 5-μl aliquot of each was directly sequenced using a Sequenase kit (U.S. Biochemical Corp., Cleveland, OH). P198, P201, and an additional internal oligonucleotide, P202 5′CTGCCGTTCCGGCCGACCAC3′ (at nt 1503 to 1522), were used as the sequencing primers.

Immunostaining

Liver biopsies were undertaken between 7 days and 3 months, 13 days after admission for acute hepatitis and during admission for chronic infection. The biopsy samples were fixed in 10% formalin, followed by paraffin-embedding, and 4-μm-thick sections were processed for hematoxylin and eosin staining and indirect immunoperoxidase staining of HCV, HBsAg, and HBc antigen (HBcAg).

Each of the three first antibodies was raised in a rabbit. The immunogen was a recombinant hepatitis C core (amino acids 1–120, Chemo-Sero-Therapeutic Research Institute, Japan) for anti-HCV, HBsAg (Medical & Biological Labs. Co. Ltd., Japan, and Eisai Col. Ltd., Japan) derived from human serum with anti-HBs, and recombinant HBcAg (Dako Japan Co. Ltd., Japan and Eisai Co. Ltd.) for anti-HBc. These three antibodies had been demonstrated previously to stain specifically each respective antigen in formalin-fixed, paraffin-embedded sections [Uchida et al., 1994b; Uchida et al., 1994c].

Deparaffinized sections were incubated with the first antibody (anti-HCV diluted 1:500 or anti-HBc or anti-HBs diluted 1:1000) at 4°C overnight, and then with the second antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (Medical & Biological Labs. Co. Ltd.) at a dilution of 1:500 at 37°C for 1 hr. The reaction products were visualized by treatment with 3,3′-diaminobenzidine tetrahydrochloride, and the sections were briefly counterstained with hematoxylin for HCV and HBsAg staining. After each step, the sections were rinsed with phosphate-buffered saline.

As a blocking control, the first antibody was absorbed before application to sections with its respective antigen (42.5 μg to 100 μg antigen in 1 ml of antibody diluted 1:500 to 1:1000) at 37°C for 1 hr, followed by clarification by centrifugation. As a control, the second antibody was applied to sections after incubation with normal rabbit serum or elimination of the first antibody reaction. Three HBsAg- and HBeAg-positive and anti-HCV-negative liver biopsy samples served as positive controls for HBsAg and HBcAg. In addition, three normal samples obtained at surgery served as negative controls for HCV, HBsAg, and HBcAg.

Cloning of the Full-Length Genome of the Silent HBV DNA Mutant and Construction of a Tandem Dimer

The total nucleotides of the silent HBV DNA were amplified as two overlapping segments (A and B) by nested PCR of a sample from patient No. 28. The PCR pair primers were P197 5′CCATACTGCGGAACTCCTAG3′ (at nt 1268 to 1287) and P210 5′GGGTTGAAGTCCCAATCTGG3′ (at nt 2976 to 2995) for the first-stage PCR and P198 5′TTTTGCTCGCAGCCGGTCTG3′ (at nt 1295 to 1314) and P211 5′AGGGTCCAACTGGTGATCGG3′ (at nt 2932 to 2951) for the second-stage PCR of Segment A. The pair primers were P191 5′GGCATTAAACCTTATTATCC3′ (at nt 2696 to 2715) and P205 5′GGTCGTCCGCGGGATTCAGC3′ (at nt 1442 to 1461) for the first-stage PCR and P192 5′ATATAAGAGAGAAACTACAC3′ (at nt 2785 to 2804) and P206 5′CCGACGGGACGTAGACAAAG3′ (at nt 1421 to 1440) for the second-stage PCR of Segment B. The PCR was performed as described previously.

Two successfully amplified segments were each double digested with *Bst*EII and *Bam*HI (Toyobo Biochemicals, Japan) after purification using the Geneclean II kit. Then the two digested segments were ligated to each other using T4 DNA ligase (Toyobo Biochemicals), followed by digestion with *Bam*HI. The linearized HBV DNA was inserted into pUC19 (Toyobo Chemicals) and then the cloned HBV DNA was used to transform *E. coli* JM109 (Toyobo Biochemicals). The resulting transformant was mass-cultured under ampicillin (50 μg/ml) pressure, followed by purification of the recombinant plasmid. This HBV DNA was termed A11 (GSDB/DDBJ/EMBL/NCBL DNA databank accession number: D50489) and was 3192 nt in length after sequencing as described above using the sequencing primers mentioned elsewhere [Uchida et al., 1994a]. A11 was revealed to have an 8-nt deletion in the X

ORF, which was the same as that previously reported in silent HBV DNAs [Uchida et al., 1994a; Uchida et al., 1995]. Subsequently, a head-to-tail tandem-repeat HBV DNA dimer was constructed from this cloned A11 at the *Bam*HI site and subcloned into the pUC9 plasmid at the *Bam*H1 site. This replication-competent dimer, named D1, was used for subsequent in vitro transfection experiments.

Cotransfection Experiments and Quantification of HCV RNA

An established cell line, HuH-7 (Nakabayashi et al., 1982), was cotransfected with the HCV RNA and with the silent HBV DNA dimer, D1. The HCV RNA was in vitro transcribed by T7 RNA polymerase from the expression vector pHCV (kindly provided by Dr. Michael Houghton) using a MEGAscript T7 kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. This HCV RNA had been previously confirmed to be replication-competent in HuH-7 cells for more than 4 weeks after transfection [Yoo et al., 1995]. As a control for D1, a wild-type HBV DNA head-to-tail dimer was prepared (named p2PYW310, kindly provided by Dr. Makoto Mayumi).

The HuH-7 cells were grown at 37°C in the presence of 5% CO_2 in RPMI medium 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum and 50 μg/ml kanamycin, then seeded into 50-ml flasks (Greiner Labortechnik, Germany). When the cells reached 40–60% confluence (approximately 5×10^6 cells in a saturation state in 5 ml of medium), they were transfected with (1) 10 μg HCV RNA alone, (2) 10 μg HCV RNA plus 6 μg D1, or (3) 10 μg HCV RNA plus 6 μg wild-type HBV DNA dimer, using Lipofectin (Life Technologies) according to the manufacturer's recommendations. As a negative control, 10 μg of HCV RNA alone was added to cell cultures without the Lipofection treatment. Each experimental group was prepared in duplicate.

After transfection for 20 hr, the supernatants were removed and new media were added. Subsequent subcultures were performed using 25% of the confluent cells (the remainder were frozen or fixed in formalin) at 7-day intervals, and the medium was changed 4 days after transfection or subculture. After transfection, culture was continued for 3 weeks. Levels of HCV RNA in the supernatants were quantified using an Amplicore HCV Monitor™ kit (Hoffmann-la-Roche, Ltd., Nutley, NJ). The transfection of wild-type HBV DNA dimer using this method results in the secretion of HBsAg into supernatant at a rate of about 3–50 ng/ml/2 days for a period of 3 weeks, whereas this HBsAg secretion is reduced about two- to five-fold in the case of D1 transfection. Immunostaining of the cell pellets after transfection show about 1% cell positivity for HBsAg and HBcAg in both HBV DNAs [manuscript in preparation], indicating that the transfection efficiency did not differ between the HBV DNAs.

RESULTS

Detection of Serum HBV DNA and Tissue Viral Antigens

Using the PCR, the specific HBV DNA was amplified in 8 (Nos. 2 and 3 were negative) of the 10 patients with acute hepatitis, 8 of the 10 patients with chronic hepatitis (Nos. 11 and 18 were negative), and all 10 patients with liver cirrhosis. The expected bands were all seen at the second-stage PCR but not at the first-stage PCR. The negative controls did not show any bands. Subsequent nt sequencing of HBV DNAs from 9 patients revealed common mutations of the X ORF, consisting of a point mutation of DR2 (*T*TCACCTCTGC to *C*TCACCTCTGC) and an 8-nt deletion between nt 1770 and 1777 (Fig. 1), which are always recognized in silent HBV mutant infections [Uchida et al., 1994a; Uchida et al., 1995]. The 8-nt deletion created a new stop codon by a frameshift and truncated the X protein from 154 to 134 amino acids.

Hematoxylin and eosin staining of biopsy samples from all the patients revealed necroinflammation, which is characterized by hepatocellular necrosis associated with lymphocytic infiltration [Uchida, 1994]. Immunostaining revealed diffuse or focal localization of HCV in the hepatocellular cytoplasm, with various intensities and distributions in 22 (73.3%) of the 30 patients (Fig. 2a).

The distribution of HBsAg was similar to that of HCV; the former was stained diffusely or focally within the hepatocellular cytoplasm in 15 (50.0%) of the 30 patients. The same hepatocytes were positive frequently for both HCV and HBsAg (Figs. 2a and b). The overall staining of HBsAg was much weaker than that of the HBsAg-positive controls. No cytoplasmic inclusion (so-called ground-glass staining) or membrane staining was present. HBcAg-positive staining was seen in a few hepatocellular nuclei in 4 (13.3%) of the 30 patients. The staining intensity and pattern of HBsAg and HBcAg were similar to those of silent HBV infection alone [Uchida et al., 1994c].

None of the patients with acute hepatitis showed staining for HBsAg and HBcAg, whereas all 10 patients with liver cirrhosis exhibited positive staining for HBsAg. The two patients with chronic hepatitis, who were negative for HBV DNA by PCR, were also negative for HBsAg staining. The data for immunostaining are shown in Table I. The control staining was negative, except for HBsAg and HBcAg in the specimens from HBsAg-positive patients. Therefore we concluded that the immunostaining was specific.

In Vitro Transfection and HCV RNA Qualification

Throughout the in vitro transfection and subsequent 3 weeks of cultivation, the HuH-7 cells grew continuously and did not exhibit any morphological alterations. The HCV RNA levels measured in the supernatants are given in Table II. The levels of HCV RNA in Groups 1–3 were higher than those of the negative con-

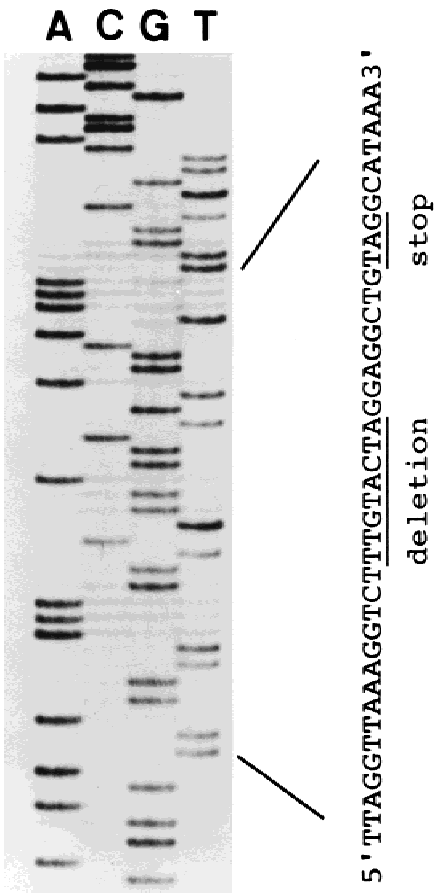

Fig. 1. Nucleotide sequencing of the distal X open reading frame of patient No. 9. An 8-nucleotide deletion has created a frameshift with a resulting stop codon downstream.

trol, indicating active HCV RNA replication in the three experimental groups. The HCV RNA titers tended to fall with time and passage number. The overall levels were highest after cotransfection with HCV RNA and the silent-mutant HBV DNA, and were to 5 times higher than after transfection with HCV RNA alone, and HCV RNA continued to be detectable for the longest period in this cotransfection group. By contrast, the levels of HCV RNA were lowest after cotransfection with HCV RNA and wild-type HBV DNA and were undetectable initially. A similar result was obtained in a separate cotransfection experiment.

DISCUSSION

The results of the present investigation using PCR strongly suggest that most (86.7%) patients with HCV infection have been infected simultaneously with HBV regardless of the HCV genotype. The high rate of HBsAg immunostaining in the liver of patients with chronic HCV infection (75%, 15 of 20) strongly supports this possibility. Moreover, this HBV had common critical mutations in the X ORF, which seem to be responsible for silent HBV infection. The complete nucleotide sequencing of HBV DNA recovered from patient No. 28 revealed mutations that are characteristic of HBV DNA recovered from patients with silent HBV infection without concomitant HCV infection [Uchida et al., 1994a; Uchida et al., 1995]. These findings indicate that the same, or a very similar, HBV mutant is the infectious agent in both silent HBV infection and HCV infection. This coinfection may not be unique to Japanese patients, since it has also been reported elsewhere [Baginski et al., 1992; Brechot et al., 1991; Chung et al., 1995; Gonzalez et al., 1995; Sardo et al., 1994]. Although the coinfection rate we observed was higher than that in the literature [Gonzalez et al., 1995; Sardo et al., 1994], a similar coinfection rate has been confirmed in two other laboratories in Japan [Dr. Ryo Fukuda and Dr. Akira Nishizono, personal communication]. Coinfection of silent HBV with HCV may thus be universal, although the rate is considered to vary among areas and countries.

All 10 liver samples from patients with acute hepatitis were negative for HBsAg by immunostaining. However, in our experience, HBsAg staining is usually weak or negative in liver samples from patients with HBsAg-positive acute hepatitis [unpublished data]. Therefore, the absence of staining seen in the present samples from patients with acute hepatitis seems to be reasonable. The two patients (Nos. 11 and 18) with chronic hepatitis who were negative for HBV DNA by PCR were also negative for HBsAg and HBcAg by immunostaining. These two patients, and the other two with acute hepatitis (Nos. 2 and 3) with negative PCR findings, may not have been infected with HBV, although a very low level of coinfection cannot be completely excluded. In any event, the present report is the first to provide immunostaining data that confirm the silent HBV coinfection with HCV. The cases of chronic hepatitis showing absence of HBsAg staining also tended to be negative for HCV staining, probably as a result of inadequate specimen preparation; staining for both antigens is usually weak and can be easily abolished by inadequate fixation or sample preparation.

Little or no expression of HBcAg indicates suppressed HBV infection, since HBcAg expression is well correlated with viral replication [Suzuki et al., 1987]. The failure of the first-stage PCR to amplify the target HBV DNA also indicated that the in vivo level of HBV DNA replication was low. Thus HBV DNA replication and expression seem to be markedly suppressed in hepatitis C, presumably as a result of the mutations in the X ORF.

Seven of the 26 patients with silent HBV infections had anti-HBs at the time of presentation or during chronic infection. This finding appears to be paradoxical, considering that anti-HBs is the neutralizing antibody and its occurrence generally indicates the elimination of HBV infection. The same finding has been reported previously for coinfections of HCV and silent HBV [Baginski et al., 1992; Brechot et al, 1991; Chung et al., 1995; Sardo et al., 1994]. It is thus probable that silent HBV can replicate by some unknown mechanism despite the presence of anti-HBs antibodies.

There are two possible ways in which coinfection

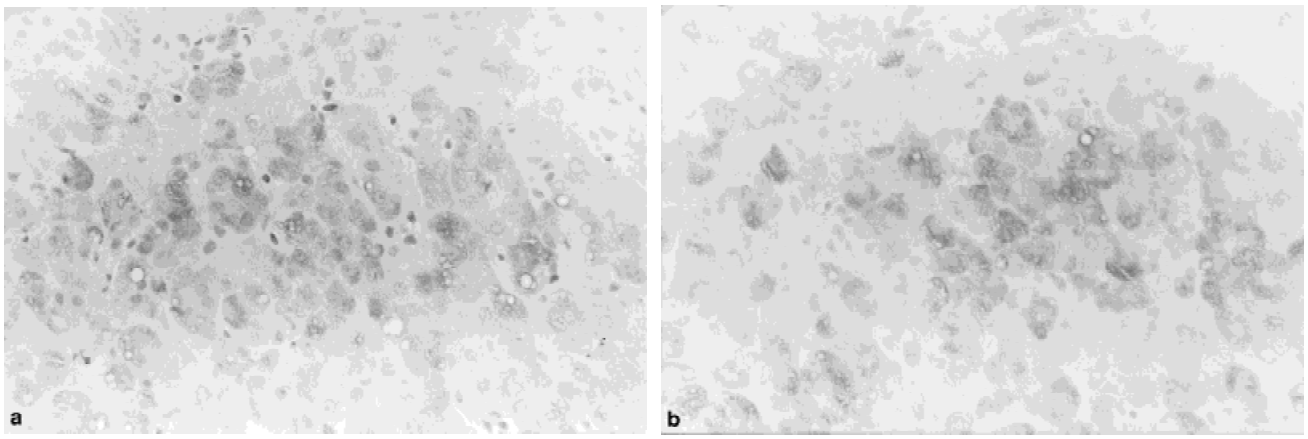

Fig. 2. Immunoperoxidase staining for hepatitis C virus **(a)** and hepatitis B surface antigen **(b)** of the same liver area in patient No. 26 (×200, counterstained with hematoxylin). Individual hepatocytes are frequently positive for the both antigens. The cytoplasm is stained diffusely or focally at various intensities.

TABLE II. Quantification of HCV RNA in Cell Culture Supernatants After In Vitro Transfection or Cotransfection*

	Days after (co)transfection					
	4	7	11	14	18	21
Group 1, HCV RNA alone	50K[a]	130K	45K	8.6K	2.3K	UD[b]
Group 2, HCV RNA + silent-mutant HBV DNA	250K	140K	60K	18K	5.7K	2.3K
Group 3, HCV RNA + wild-type HBV DNA	32K	47K	17K	2.1K	UD	UD
Negative control	31K	29K	3K	UD	UD	UD

*The experiment was performed in duplicate and the value given is the average. The levels of HCV (hepatitis C virus) RNA were measured using an Amplicore HCV Monitor™ kit (Hoffmann-la Roche, Nutley, NJ) for culture supernatants. For each transfection the following amounts were used: 10 μg for hepatitis C virus (HCV) RNA and 6 μg each for silent-mutant and wild-type hepatitis B virus (HBV) DNA. In the negative control, 10 μg of HCV RNA alone was added to the cell culture without the Lipofectin treatment.
[a]K = 10^3 copies HCV RNA/ml.
[b]UD = undetected (less than 1K).

with HBV and HCV can occur in the same liver. One is incidental; since HCV and HBV are transmitted by the same route, patients may be infected with the two viruses simultaneously or sequentially. The other possibility is that one virus assists infection with and/or sustains the replication of the other virus in hepatocytes. If so, the silent HBV mutant is assumed to assist infection by and/or maintain the replication of HCV, because the former can infect and replicate alone, as seen in cases of infection by silent hepatitis B where HCV is not present. In order to test this hypothesis, we carried out in vitro cotransfection experiment, and the results showed that the level of HCV RNA released from transiently transfected cells was higher in the group cotransfected with HCV RNA and silent-mutant HBV DNA than in the group transfected with HCV RNA alone. By contrast, wild-type HBV DNA suppressed the secretion of HCV in the cotransfection experiment.

The difference in the secretion of HCV RNA may not be due to the transfection efficiency, at least between wild-type and silent-mutant HBV DNAs, because their transfection efficiencies did not differ as described in Materials and Methods. Although wild-type HBV DNA suppressed HCV RNA secretion in the cotransfection experiment, HCV RNA also suppressed HBsAg secretion about four- to six-fold in comparison with either of the HBV DNA transfections alone. Also, immunostaining of cell pellets revealed a decrease in HBcAg-positive cells more than five-fold in the cotransfection experiments in comparison with either of the HBV DNA transfections alone (data not shown). This phenomenon suggests that HBV RNA suppressed HBV DNA replication, since HBcAg expression is well correlated with viral replication [Suzuki et al., 1987].

Generally, it is thought that two hepatitis viruses interfere with each other if they infect the same subject [Koike et al., 1995; Liaw, 1995]. Suppression of HBV replication by HCV in HuH-7 cells has also been reported previously [Shih et al., 1993]. Considering these finding of mutual suppression in the transfection of two kinds of viruses, the enhancement of HCV RNA secretion by silent-mutant HBV DNA seems to be exceptional and noteworthy. It is probable that the promotion of HCV replication by silent-mutant HBV is greater in the livers of patients than in this in vitro system. Clarification of the mechanism involved will be important for future investigation.

Finally, we should address the putative role of silent HBV in hepatocarcinogenesis in chronic hepatitis C with or without cirrhosis. Liver infected with HCV seems to show a higher frequency of hepatocellular carcinoma (HCC) than that infected with HBsAg-positive HBV, at least in Japan, based on clinical observations

and the high rate and multicentricity of tiny HCCs or borderline lesions in livers resected because of large HCCs in patients with chronic hepatitis C [Uchida, 1995]. The sustained repeated cycles of necrosis and regeneration of hepatocytes by necroinflammation alone can not explain this phenomenon. Thus it seems reasonable to consider that the coinfecting silent HBV plays a crucial role in the hepatocarcinogenesis of chronic hepatitis C. If silent HBV promotes HCV replication via its unknown gene product(s), such product(s) may also activate or repress cellular gene(s) related to carcinogenesis. The most recent work of Koike et al. [1996] has revealed a high frequency (about 90%) of PCR amplification of HBV DNA in HCC samples from patients with anti-HCV-positive/anti-HBc-positive/HBsAg-negative status. This finding appears to support our assumption. Clarification of this hypothetical mechanism of hepatocarcinogenesis awaits further study.

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REFERENCES

Baginski I, Chemin I, Hantz O, Pichoud C, Jullien AM, Chvre JC, Vitvitski LL, Sninsky JJ, Trep C (1992): Transmission of serologically silent hepatitis B virus along with hepatitis C virus in two cases of posttranfusion hepatitis. Transfusion 32:215–220.

Brechot C, Kremsdorf D, Paterlini P, Thiers V (1991): Hepatitis B virus DNA in HBsAg-negative patients. Molecular characterization and clinical implications. Journal of Hepatology 13:S49–55.

Chung H-T, Lai C-L, Lok ASF (1995): Pathogenic role of hepatitis B virus in hepatitis B surface antigen-negative decompensated cirrhosis. Hepatology 22:25–29.

Feitelson M, Lega L, Guo J, Resti M, Rossi ME, Azzari C, Blumberg BS, Vierucci A (1994): Pathogenesis of posttransfusion viral hepatitis in children with β-thalassemia. Hepatology 19:558–568.

Fukuda R, Ishimura N, Kushiyama Y, Moriyama N, Ishihara S, Chowdhury A, Tokuda A, Sakai S, Akagi S, Watanabe (1996): Hepatitis B virus with X gene mutation is associated with majority of serologically "silent" non-B, non-C chronic hepatitis. Microbiology and Immunology 40:481–488.

Gonzalez S, Navas S, Madejon A, Bartolome J, Castillo Moraleda G, Martin J, Marriott E, Herrero M, Carreno V (1995): Hepatitis B and D genomes in hepatitis B surface antigen negative patients with chronic hepatitis C. Journal of Medical Virology 45:168–173.

Koike K, Yasuda K, Yotsuyanagi H, Moriya K, Hino K, Kurokawa K, Iino S (1995): Dominant replication of either virus in dual infection with hepatitis viruses B and C. Journal of Medical Virology 45: 236–239.

Koike K, Nakamura Y, Kobayashi M, Takada S, Urashima T, Saigo K, Kobayashi S, Isono K, Hayashi I, Fujii A (1996): Hepatitis B virus DNA integration frequently observed in the hepatocellular carcinoma DNA of hepatitis C virus-infected patients. International Journal of Oncology 8:781–784.

Liang TJ, Baruch Y, Ben-Porath E, Enat R, Bassan L, Brown NV, Rimon N, Blum HE, Wands JR (1991): Hepatitis B virus infection in patients with idiopathic liver disease. Hepatology 13:1044–1051.

Liaw Y-F (1995): Role of hepatitis C virus in dual and triple hepatitis virus infection. Hepatology 22:1101–1108.

Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J (1982): Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Research 42:3858–3863.

Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Mayumi M (1992): Typing hepatitis V virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. Journal of General Virology 73:673–679.

Preisler-Adams S, Schlayer H-J, Peters T Gerok HW, Rasenack J (1993): Sequence analysis of hepatitis B virus DNA in immunologically negative infection. Archives of Virology 133:385–396.

Sardo MA, Rodino G, Brancatelli S, Pernice M, Campo S, Squadrito G, Russo F, Raimondo G (1994): Inapparent "wild-type" and "e-minus variant" HBV infection in patients with HCV-related chronic hepatitis. Liver 14:241–244.

Shih C-M, Lo SJ, Miyamura T, Chen S-Y, Lee Y-H W (1993): Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. Journal of Virology 67: 5823–5832.

Simmonds P, McOmish F, Yap PL, Chan S-W, Lin CK, Dusheiko G, Saeed AA, Holmes EC (1993): Sequence variability in the 5′ noncoding region of hepatitis C virus: identification of a new virus type and restriction on sequence diversity. Journal of General Virology 74:661–668.

Suzuki K, Uchida T, Shikata T (1987): Histopathological analysis of chronic hepatitis B virus (HBV) infection in relation to HBV replication. Liver 7:260–270.

Uchida T (1994): Pathology of hepatitis C. Intervirology 37:126–132.

Uchida T (1995): Small hepatocellular carcinoma: its relationship to multistep hepatocarcinogenesis. Pathology International 45:175–184.

Uchida T, Aye TT, Shimojima M, Gotoh K, Shikata T (1994a): Full-length nucleotide sequence of a hepatitis B virus (HBV) mutant isolated from a patient with acute hepatitis who did not exhibit serological markers for HBV infection. International Hepatology Communications 2:70–73.

Uchida T, Shikata T, Tanaka E, Kiyosawa K (1994b): Immunoperoxidase staining of hepatitis C virus in formalin-fixed, paraffin-embedded needle liver biopsies. Virchows Archiv 424:465–469.

Uchida T, Shimojima S, Gotoh K, Shikata T, Mima S (1994c): Pathology of livers infected with "silent" hepatitis B virus mutant. Liver 37:126–132.

Uchida T, Gotoh K, Shikata T (1995): Complete nucleotide sequences and the characteristics of two hepatitis B virus mutants causing serologically negative acute or chronic hepatitis B. Journal of Medical Virology 45:247–252.

Yoo BJ, Selby MJ, Choe J, Suh BS, Choi SH, Joh JS, Nuovo GJ, Lee H-S, Houghton M, Han JH (1995): Transfection of a differentiated human hepatoma cell line (Huh7) with in vitro-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV. Journal of Virology 69:32–38.